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CONTRACTING ORGANIZATION: Northwestern University, Evanston, IL 60208

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14. ABSTRACT
During the first reporting period my investigations on Fas (also called CD95) signaling in breast cancer and in breast cancer stem cells (BCSCs) led me to identify a novel life-protective role for Fas. Briefly, I found that the non-BCSCs component of a breast tumor is more sensitive to Fas-mediated apoptosis, while the BCSCs part is more sensitive to the death induced by the elimination of CD95 (a phenomenon we have recently described and named DICE). This was found in different breast cancer cells lines and further investigations will provide the basis for the identification of novel molecular targets for the treatment of breast cancer. During this first reporting period I have in fact observed a significant enhancement of cancer cell death by simultaneously inducing apoptosis and DICE in breast cancer cells, with many potential therapeutical applications. I could also demonstrate the involvement of miRNA in the process. Moreover, I have developed a novel plasmid-based tool to isolate BCSCS by the activity of miRNAs, and I am going to optimize and test the relevance of its use in the next reporting period.

15. SUBJECT TERMS
Fas, FasL, Cancer, Cancer Stem cells, Apoptosis, miRNA, EMT, cell death.

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1. INTRODUCTION

Background. It was in breast cancer that cells with the properties of cancer stem cells (CSCs), believed to be responsible for some of the most lethal features, including chemo-resistance and relapse, were first discovered. Targeting of CSCs has been recognized as a very promising strategy to treat tumors, but little is known about the molecular mechanisms that regulate CSCs and possible molecular targets to eliminate them. The epithelial-to-mesenchymal transition (EMT) is a process that leads to the development of breast CSCs, and it is regulated by micro(mi)RNAs. Fas is a death receptor mostly known as an inducer of apoptosis. It is however emerging that Fas can also mediate survival signals. ***Hypothesis:*** Our group has several pieces of evidence to suggest that knockdown of Fas or its ligand FasL in cancer cells causes metabolic stress eventually resulting in the death of the cells (now published in Hadji et al. Cell Reports 2014). Based on my preliminary data I hypothesized that breast CSC and non-CSCs have differential sensitivity to Fas stimulation and knockdown. I tested this hypothesis in different models and with different approaches.

2. KEYWORDS

Fas, FasL, Cancer, Cancer Stem cells, Apoptosis, miRNA, EMT, cell death.

3. ACCOMPLISHMENT

Major goals of the project.

The project was divided in major goals as follows:

Task 1. Test if Fas has opposite activities in CSCs and non-CSCs
Task 2. Determine if the activity of Fas in breast CSCs is connected to the expression of miRNAs
Task 3. Investigate if ablation of Fas or FasL helps to break therapy resistance by killing CSCs

Subtasks 1a to 1d have been completed in the first 8 months.
Subtask 1e has not been performed yet.
Subtask 1f has not been performed yet.

Subtasks 1a to 1d have been completed in the first 12 months.
Subtask 2e has not been performed yet.
Subtask 2f has not been performed yet.

Task 3. Has not been performed yet.

Major accomplishments.

Task 1.

1a) and 1d) We used the two breast cancer cell lines MCF-7 (epithelial like, with low CSCs content) and MDA-MB-231 (mesenschymal-like, high CSCs content, see **Figure 1**) to test the sensitivity to Fas (CD95) induced apoptosis induced by treatment with either an anti-Fas agonistic antibody (APO-1) or the soluble Fas ligand (Lz-FASL or Lz-CD95L). This was assayed with trypan blue exclusion assay and cell count at the microscope. MDA-MB231 cells showed to be resistant to Fas-mediated apoptosis (**Figure 1**). Moreover, MCF-7 and T47D showed to have an increase in the CSCs

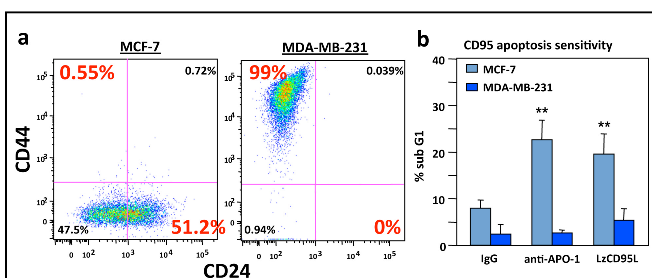

Figure 1. a) FACS plots of MCF-7 and MDA-MB-231 cells stained with CD44 and CD24. b) Fas-mediated apoptosis sensitivity of MCF-7 and MDA-MB231 cells. All experiments were repeated three times, data are ± standard deviation and p-values calculated with t-tests.

population (characterized as cells with lowCD24 and highCD44 expression) when they were stimulated with anti-APO-1 for a prolonged period of time (2 weeks). The cells were also subjected to sphere forming assay (a functional readout for the CSCs content) for one additional week and spheres that formed were counted on a microscope, see **Figure 2**. This confirmed the enrichment in CSCs induced by Fas stimulation.

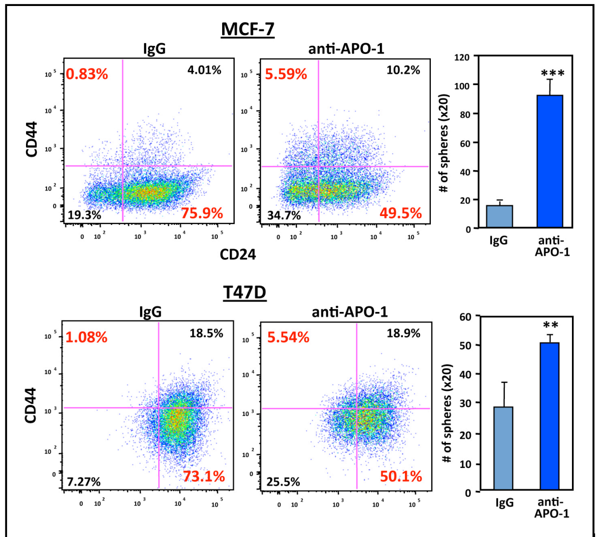

Figure 2. FACS plots of MCF-7 and T47D treated for 2 weeks with anti-Apo-1 or with control antibody stained for CD24 and CD44 (left) and seeded for spheres formation assay (quantification of spheres formed after one additional week on the right). All experiments were repeated three times, data are ± standard deviation and p-values calculated with t-tests.

1b) MCF-7 and MDA-MB-231 cells were infected with the lentiviral shRNA particles targeting FasL (pLKO-L3) and the cell death was assayed with PI staining of lysed nuclei (Nicoletti staining assay). This experiment showed that the cells with higher CSCs (MDA-MB-231) content have higher sensitivity to the death induced by CD95/CD95L elimination, or DICE (see Hadji et al. Cell Reports 2014) as achieved by the infection with the infection with the pLKO-L3 virus targeting FasL, see **Figure 3**. This was assayed by PI staining of isolated nuclei (analysis of subG1 fraction). Moreover, MCF-7 and T47D showed to have a reduced CSC population upon DICE induction, **Figure 4**. Finally, the combination of Fas stimulation and knockdown showed to synergistically kill MCF-7 cells, and this was proved to be just partially dependent of the Fas surface content upon DICE (**Figure 5**. and insert). These results indicate that the combination of CSCs and non-CSCs targeting by the combination of Fas stimulation and knockdown could represent an effective strategy to eradicate breast tumors.

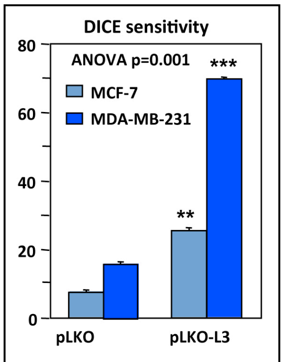

Figure 3. DICE sensitivity of MCF-7 and MDA-MB-231 cells one week after infection with either pLKO or pLKO-L3 virus.

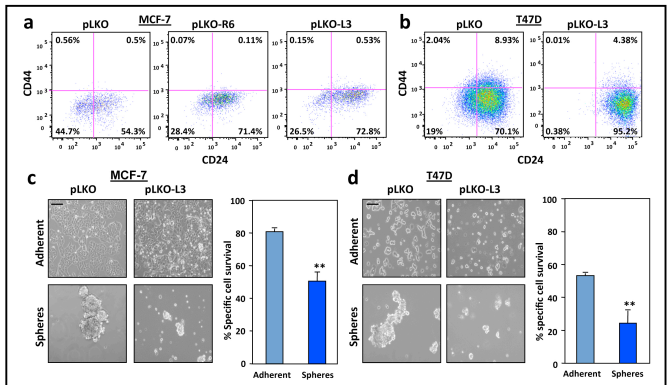

Figure 4. FACS plots of a) MCF-7 and b) T47D cells 10 days after infection with indicated virus (R6 targeting Fas and L3 targeting FasL) and stained for CD24 and CD44. c) MCF-7 and d) T47D cells were also plated in adherent and sphere-forming conditions and the survival analyzed after 4 days by FACS.

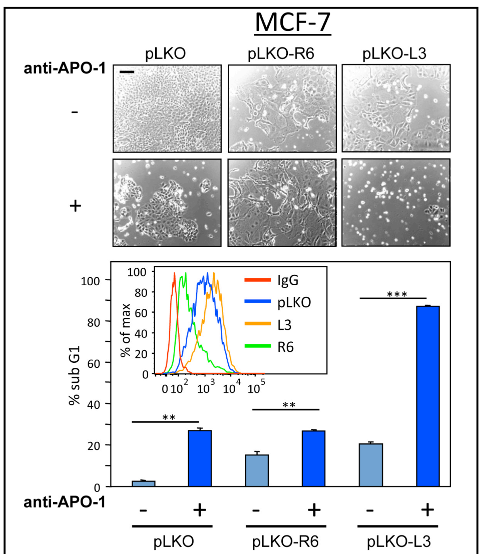

Figure 5. Synergistic combination of Fas/FasL knockdown and Fas stimulation by anti-APO-1 in MCF-7 cells. Cells were treated one week after infection with the indicated viruses and the death assayed by SubG1 quantification. Inset shows Fas expression in all condition at the time of the treatment. All experiments were repeated three times, data are ± standard deviation and p-values calculated with t-tests.

1c) CFSE was used to label MCF-7 cells treated as in 1a), to quantify net cell death and cell growth in the culture at the same time. **Figure 6** shows the results with in the X-axis the amount of dye incorporated in the population of cells, which is inversely proportional to the number of cell divisions (proliferation rate). This experiment was important because showed that apoptosis induction with Fas stimulation promotes the emergence of a slowly-proliferating population of cells, which was found out to have a CSC phenotype (CD44+), rather than stimulating they growth thus indicating an effect on cell differentiation. The experiment was not performed with the conditions as in 1b, mainly because of the longer time of incubation needed for the cells infected with the pLKO-L3 to be affected in terms of growth and survival (by then the cells would have lost the dye).

1e) A preliminary experiment of orthotropic injection of breast cancer cells has been performed, using the protocol established by Dr. Koblinski and colleagues in MDA-MB-231 cells stably expressing a dox-inducible inducible sh-FASL virus (Hadji et al. Cell Reports 2014). A total of 12 mice were used. Mouse have been treated with dox for total three weeks, the mice were sacrificed and tumor collected. We could observe a moderate reduction in growth in cells infected with the sh-FASL virus in the presence of dox, as well as an increased number of dead cells, in line with the hypothesis of a life-preserving function of Fas. We are now planning to repeat the experiment with a prolonged dox treatment and with more mice, which will include an immunohistochemical analysis of CSCs markers on harvested tumors.

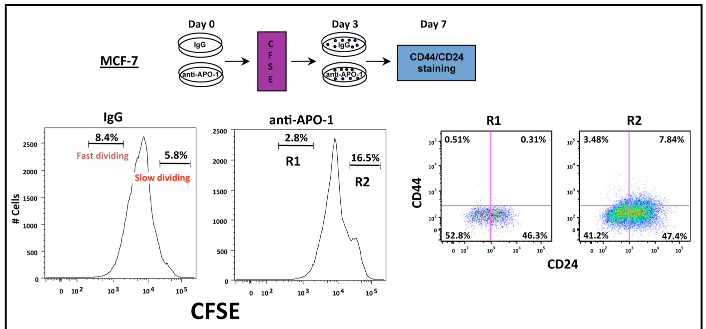

Figure 6. *Up.* Outline of the experiment. *Bottom.* FACS plots of CFSE-labeled MCF-7 cells treated with anti-APO-1. R1 and R2 gates show fast and slowly proliferating cells, respectively. The FACS plots show the staining with CD24 and CD44 of gated cells.

Task 2.

2a) MCF-7 were found highly expressing miR-200c, and therefore miR-200c was overexpressed in MDA-MB-231, which express miR-200 at very low level. MDA-MB-231 cells overexspressing miR-200c were subjected to FasL knockdown, and this resulted in reduced CSC properties. Importantly, death assay by PI staining showed that this treatment could significantly slow down and prevent DICE (**Figure 7**). This treatment however did not sensitize cells to Fas-induced apoptosis. Let7 was not investigated, as our results did show that miR-200 members were more strongly connected with the properties of breast CSCs.

2b) The screening has been performed, and miR-221 was identified as a breast CSCs miRNA. Cells overexpressing miR-200c were in fact found to suppress CSCs function and CSCs markers and were found to reduce miR-221 levels (**Figure 8)**, while cells with miR-200c knockdown showed an increase in miR-221 levels.

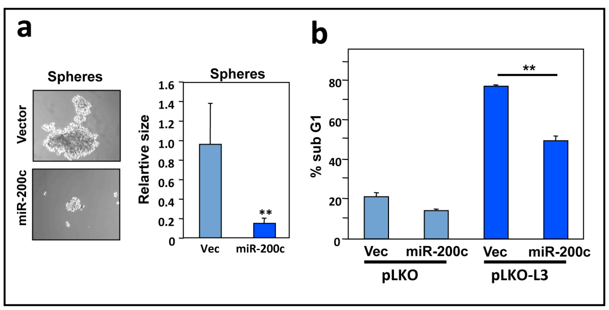

Figure 7. a) Representative pictures of sphere formed from MDA-MB-231 vector and miR-200c overexpressing cells, with quantification of the size. b) DICE sensitivity of MDA-MB-231 vector and miR-200c overexpressing cells one week after infection (percentages of SubG1 cells).

3c and 3d) Some of the most relevant miRNA sensors have been cloned. Specifically the single-activity miR-200 sensor has been found as the most powerful to detect and isolate breast CSCs. The green-red configuration (on a double CMV promoter), with green (GFP) carrying the mutated target region and with red (DsRed) carrying the wild-type sequence, was found to be the best performing in terms of sensitivity. Sensors were tested by transient overexpression of the in 293T cells in the presence/absence of the corresponding pre-miRNAs or miRNA inhibitors to validate the efficacy/specificity of targeting by flow-cytometry. I confirmed that the miRNA sensor was selective for miR-200 (b/c family members). Then, the miRNA sensor was converted in a lentiviral vector and tested in MCF-7 cells. I could confirm that the sensor detected changes at endogenous expression levels, and that CD44high/CD24low CSCs from breast cancer MCF-7 and T47D cells could be enriched by using the miRNA sensor (**Figure 9**).

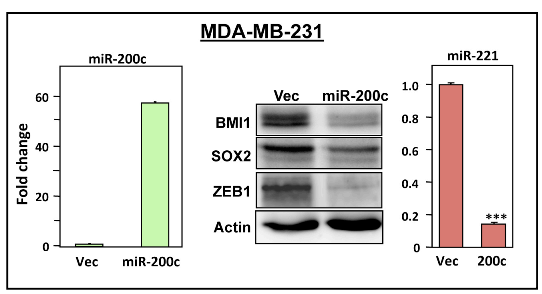

Figure 8. Quantitative PCR on miR-200c (left) and miR-221 (right) expression levels in MDA-MB-231 vector and miR-200c overexpressing cells. The western blot shows the reduction of breast CSC markers upon miR-200c overexpression.

Opportunities for training and professional development.

In this first reporting year I had the opportunity to be trained in several techniques and learn novel concepts in molecular and cellular biology. For example, I gained experience in the mechanisms of cell death and of tumor progression. I started gaining experience with mouse work (various type of injections, limited dilutions in nude mice and other mouse models) and I had the opportunity to work and be trained in the field of breast cancer stem cells research (learning technique like flow-cytometry in breast surface markers, sphere-formation assay and orthotropic injection of breast cancer cells in the mammary fat pad of nude mice). Opportunities for professional development included, among other seminars and meeting, the participation to the 15th Annual Lynn Sage Breast Cancer Symposium, held in Chicago September 26-29 2013, and to present my data at the poster session of the 10th Annual Lewis Landsberg Research Day which happened April 3 on the Chicago campus at Northwestern University .

Results dissemination.

An opportunity of result dissemination will soon be available during the month of November, as an official press release from the Northwestern University Feinberg School of Medicine will be issued accompanying and commenting the publication of the *Nature Communications* paper (see below).

Plans for the next reporting period.

The plan for the next reporting period will be to complete Task 1 and Task 2 and to start the preliminary experiments necessary for the Task 3 with the goal of understanding whether the increased sensitivity of CSCs to DICE, could be used to break the therapy resistance of breast cancers and to dissect the molecular determinants of this novel form of death in the context of breast cancer. Importantly, the lab has recently acquired an IncuCyte™ Kinetic Live Cell Imaging System from Essen Bioscience, which allows a variety of biological assays, and which is especially useful for the analysis of the efficacy of drug combinations and chemoresistance. The cells will be therefore stimulated through Fas or infected with the shRNA viruses suppressing Fas/FasL expression, in combination with a pool of chemotherapetic drugs used in the treatment of breast cancer or with potential new drugs (like small molecule inhibitors that will be found in the small-molecule screening). Sequential images of each of the 96-wells or 24-wells will be taken automatically, and a software will monitor in real-time several parameters like confluency, cell morphology, cell death (with the use of the fluorescent marker YOYO-1), as well as invasion, migration and other functional assays. I have recently used the Incucyte machine for a preliminary experiment measuring the etoposide sensitivity of breast cancer cells MCF-7. The cells were seeded with increasing concentrations of etoposide and the confluence has been measured by the software based on the images from the pictures taken every 2 hours for an extended period of time, see **Figure 10**. The results showed as expected a dose-dependent inhibition of growth. This machine will help to perform the experiments in all Tasks and, particularly the Task 3 when combining DICE with several chemotherapeutic treatments. In addition to this, in light of my recent training on orthotropic mouse injection (in collaboration with Dr. Koblinsky) I will consider the possibility to expand and validate the observations from the Task 3 in mice. This will probably require an amendment to the existing animal protocol.

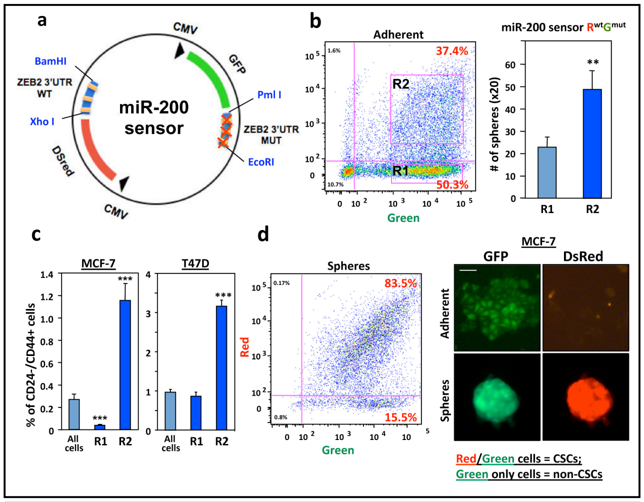

Figure 9. a) Schematic of the lentiviral sensor plasmid. b) FACS plots of MCF-7 cells infected with the miR-200 DsRedwtGFPmut sorted and grown in sphere-forming conditions (quantifications on the left). c) Percentages of CD24lowCD44high in MCF-7 and T47D infected with the sensor in the gated regions. d) FACS plots and representative images of MCF-7 cells infected with the miR-200 DsRedwtGFPmut and grown in adherent and in sphere-forming conditions. Note the mammospheres acquiring red fluorescence, indicating the loss of miR-200 expression

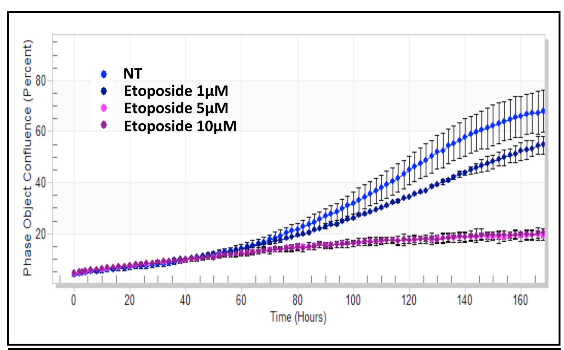

Figure 10. Confluency of MCF-7 cells either untreated or treated with indicated doses of etoposide, as assessed by the Incucyte machine over an extended period of time.

4. IMPACT

Impact on breast cancer research.
I expect the findings to have a profound impact on the scientific community in the field of breast cancer research. It is in fact commonly acknowledged that approaches aiming at killing cancer cells through conventional therapies have only a limited potential, and that new therapies need to be implemented from the recent advances made in basic cancer research. The breast cancer research field has been one of the first for which the impact of CSCs has been shown and the molecular determinants driving breast CSCs have been under extensive investigation. In this reporting period, I have provided evidences that the anti-apoptotic activity of Fas are strongly connected with the survival and the functions of breast CSCs, challenging the common view of Fas as a classical tumor suppressor gene in cancer, and paving the way for many translational applications. This lab (and maybe others in the future) will investigate novel strategies to apply these findings with the aim to deliver DICE in cancer patients, and in the next reporting period we will understand the possible impact of the combination with the conventional chemotherapy or with new small-molecule inhibitors, which may allow us to maximize the killing of breast tumors. Moreover, the development of miRNA sensors allowing us to monitor the formation of breast CSCs in living cells could represent a great tool for the breast cancer research community.

Impact on other research fields.
These findings, and especially regarding the DICE sensitivity and the miRNA sensors, could have a profound impact also on the research of other cancer types. We have, for example data that also colorectal, ovarian, liver and other cancers from a variety of origins have similar features, suggesting that the findings have applications beyond the field of breast cancer.

Impact on other research fields.
Nothing to report.

Impact on society and beyond science.
Nothing to report.

5. CHANGES/PROBLEMS

There are no major changes or problems to report in this reporting period. There are no changes to report in terms of approach and no anticipated problems or delays, or changes with a significant impact on expenditures.

6. PRODUCTS

A paper entitles "CD95 and CD95L promote and protect cancer stem cells" has been recently accepted for publication in the journal *Nature Communications* (see below the letter of acceptance). The authors are Paolo Ceppi, Abbas Hadji, Frederick J. Kohlhapp, Abhinandan Pattanayak, Annika Hau, Xia Liu, Huiping Liu, Andrea E. Murmann & Marcus E. Peter. All authors are from Northwestern University, except for Xia Liu and Huiping Liu (collaborators from CaseWestern Reserve University in Celeveland) which helped with the establishment of a patient-derived mouse model of breast cancer used in the paper. This work was funded by this DOD postdoctoral fellowship, Northern Ohio Golf Charities & Foundation and R00 CA160638 (to H.L.), and a Northwestern Memorial Foundation-Lynn Sage Cancer Research Foundation grant and R01 CA149356 (to M.E.P.).

7. PARTECIPANTS AND OTHER COLLABORATING ORGANIZATIONS

No changes in the personnel.

The Liu lab at the Department of Pathology of the CaseWestern Reserve University in Cleveland collaborated to perform the experiment on the patient-derived mouse model of breast cancer. The experiments were performed by the Principal Investigator and by the collaborators at their site on a collaboration agreement, with no costs for this project other than travel expenses for the Principal Investigator. The results of this experiment are showed in the Ceppi et al. *Nature Communications* paper currently in press, and confirmed in a primary breast cancer cell line maintained in mouse the results as shown in the Figure 4.

8. SPECIAL REPORTING REQUIREMENTS

Not applicable

9. APPENDICES

Decision letter for the Nature Communications paper:

Dear Dr Ceppi,

We are delighted to accept for publication the manuscript "CD95 and CD95L promote and protect cancer stem cells" by Paolo Ceppi, Abbas Hadji, Frederick Kohlhapp, Abhinandan Pattanayak, Annika Hau, Xia Liu, Huiping Liu, Andrea Murmann, and Marcus Peter [Paper #NCOMMS-14-03073B], on which you were a Contributing Author.

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